Industrial Crops and Products 43 (2013) 150–158

Contents lists available at SciVerse ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Production and optimization of L-asparaginase from *Cladosporium* sp. using agricultural residues in solid state fermentation

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ARTICLE INFO

Article history:
Received 11 May 2012
Received in revised form 12 July 2012
Accepted 15 July 2012

Keywords:
L-Asparaginase
CCRD
Solid state fermentation (SSF)
Cladosporium sp.
Agro-industrial residues

ABSTRACT

The present communication deals with the production of L-asparaginase from *Cladosporium* sp. in solid state fermentation (SSF) using agro-industrial residues. When used as sole source for growth in SSF, wheat bran supported maximum enzyme production by *Cladosporium* sp. followed by rice bran and bagasse. Central composite rotatable designs (CCRDs) with five process variables were studied each at levels −2, −1, 0, +1 and +2. A 120 h fermentation time under aerobic condition optimized using response surface methodology (RSM) indicated that with moisture content of 58%, pH of 5.8, incubation temperature of 30 °C appeared optimal for enzyme production by SSF. Enzyme yield (3.74 U) was maximum at these optimized conditions. Presence of Tween 20 enhanced enzyme production by 1.3 folds. A bed height of 3 and 5 cm was appropriate to obtain high enzyme production. The results optimized could be translated to 1 kg tray fermentation. The study suggested that choosing an appropriate substrate when coupled with process level optimization improves enzyme production markedly. Developing an L-asparaginase production process based on wheat bran as a substrate in SSF is economically attractive as it is a cheap and readily available raw material in agriculture-based countries. This result is of significant interest due to the low cost and abundant availability of residues.

1. Introduction

L-Asparaginase (E.C.3.5.1.1) is present in a wide range of organisms including animals, microbes, plants, and in the serum of certain rodents but not in human beings (Bessoumy et al., 2004). Although L-asparaginase has been found in various plant and animal species, due to the difficulty in extraction procedure of this enzyme, other potential sources like microorganisms were screened. Microorganisms like fungi and bacteria have proved to be very efficient and inexpensive sources of this enzyme. L-Asparaginase is produced by a number of microorganisms including *Escherichia coli*, *Erwinia cartovora*, and some species of *Enterobacter* (Bessoumy et al., 2004). Production of this enzyme has also been reported from *Corynebacterium glutamicum* (Mesas et al., 1990), *Cylindrocarpon obtusisporum* (Raha et al., 1990), *Pseudomonas stutzeri* (Manna et al., 1995), *Rhodosporodium toruloids* (Ramakrishnan and Joseph, 1996), *Tetrahymena pyriformis* (Triantafillou et al., 1998), *Pseudomonas aeruginosa* 50071 (Bessoumy et al., 2004), *Aspergillus tamarii* and *Aspergillus terreus* (Moura et al., 2004).

L-Asparaginase production throughout the world is carried out mainly by submerged fermentation (SmF). This technique however, has many disadvantages. For instance, it is cost intensive and has low product concentration. In addition, it generates excess of effluents and consequently needs handling and disposal of large volumes of waste water during downstream processing (Bessoumy et al., 2004). Solid state fermentation (SSF) is a very effective technique as the yield of the product is many times higher than in submerged fermentation (Arima, 1964). The use of solid state fermentation process is being reported by several researchers as an alternative to submerged fermentation (Hang and Woodams, 1986; Tran et al., 1998). SSF offers many advantages over SmF such as lower energy requirements, less risk of bacterial contamination, less waste water generation and less environmental concerns regarding the disposal of solid waste (Doelle et al., 1992). Other advantages include ease of product extraction that does not require complicated methods of treating the fermented residue (Lonsane et al., 1985). In comparison with SmF, SSF offers better opportunity for the biosynthesis of low volume-high cost products (Balakrishnan and Pandey, 1996). L-Asparaginase production in SSF has been reported earlier on soy bean meal (Bessoumy et al., 2004; Abdel and Olama, 2002) and wastes from three leguminous crops-bran of *Cajanus cajan*, *Phaseolus mungo* and Glycine max (Mishra, 2006).

The traditional one-factor at a time technique used for optimizing a multivariable system is not only time consuming, but also

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http://dx.doi.org/10.1016/j.indcrop.2012.07.023

often easily misses the alternative effects between components. Also, this method requires carrying out a number of experiments to determine the optimum levels when the interactions are significant. These drawbacks of single factor optimization process can be eliminated by optimizing all the affecting parameters collectively by Doehlert experimental design (Doehlert, 1970), using response surface methodology (RSM). Recently, many statistical experimental design methods have been employed in bioprocess optimization. Basically, this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, predicting its response and checking the adequacy of the model. Several researchers in biotechnology have applied these techniques for optimization of different parameters (Francis et al., 2003; Bandaru et al., 2006; Singh and Satyanarayana, 2006; Sita and Narasimha, 2010). The objective of the present work was to optimize the medium composition and operating conditions for the enhanced production of L-asparaginase from *Cladosporium* sp. using wheat bran which is a cheap agricultural byproduct in solid state medium using RSM.

2. Materials and methods

2.1. Substrate

Different agro-industrial wastes such as wheat bran, rice bran, bagasse, paddy straw, ragi straw, corn pith, maize straw, maize cob, dry leaves, were procured from a mills at Mysore and Davanagere, Karnataka, India. These were dried at 60 °C for 72 h to reduce the moisture content to around to 5% and ground to 2–3 mm size, milled and passed through a sieve shaker. The straw fractions of the size measuring about 1 mm were used for the enzyme production studies. All the chemicals used in the present study were of analytical grade and procured from Sigma–Aldrich (St. Louis, MO, USA) and Pharmacia, Uppsala, Sweden.

2.2. Microorganisms and inoculums

70 fungal cultures used in the screening trials were isolated from soil, to which different agro-industrial wastes were mixed and left for microbial action for one to three months. These cultures were purified by dilution plating technique on Potato-dextrose-agar medium and were maintained on the same medium as slants at 4 ± 1 °C. For experimentation purposes, spore suspension was prepared by incubating the cultures on PDA plates at 30 °C for about 5–6 days, until sufficient sporulation was observed. The spores were harvested using 0.1% Tween 80 solution (*v*/*v*) and the spore count of about 1×10^7 cells/ml was used for inoculation purposes. Sub culturing was done once in 30 days.

2.3. Preparation of solid substrates

Five grams of the selected substrate was taken separately in 100 ml Erlenmeyer flasks and moistened with acidified water of pH 5.5 (60% moisture), unless otherwise specified. The contents were thoroughly mixed and the flasks were sterilized at 15 lbs pressure, 121 °C for 45 min, cooled well to room temperature before use.

2.4. Plate assay for evaluation of L-asparaginase production

Modified Czapek Dox's medium was supplemented with 0.3 ml of 2.5% phenol red dye prepared in ethanol at pH 6.5 with L-asparagine incorporated in the medium for evaluation of L-asparaginase activity. The media was autoclaved and the plates were inoculated with 3 day old culture of fungal isolates. The clear zone appeared after 48 h of growth. Uninoculated media served as control.

2.5. Inoculation and fermentation

The fungal isolates which showed clear zone in plate assay were used for screening trials. Sterilized solid media (prepared as given above) were inoculated with 1 ml of spore suspension prepared by suspending spores of a 7 day old slant in 10 ml sterile Tween 80 water. The spores and the medium were properly mixed with each other with the help of sterilized spatula and spread in the flasks and incubated at ambient temperature (26–28 °C) for 5 days. The experiments were performed in triplicate and the results described are mean of three replicates.

2.6. Extraction of the enzyme

The moldy substrate was withdrawn periodically at 24 h in aseptic condition and air dried under shade. 1 g of dry moldy substrate was taken in a beaker and distilled water (1:10) was added to it. The contents of flasks were allowed to have contact with water for 1 h with occasional stirring. The extract was filtered through Whatman No. 1 filter paper. The clear extract was centrifuged. The supernatant was used as enzyme preparation. Thus prepared crude enzyme was used for assay.

2.7. Quantitative assay for L-asparaginase activity

Quantitative determination of L-asparaginase was carried out in two types, by quantifying amount of ammonia released and by estimating the formation of β-aspartyl hydroxamate (Drainas et al., 1977). Assay of enzyme for release of ammonia was carried out as per Mashburn and Wriston (1964), 1.7 ml of 0.01 M asparagine was taken in a test tube, 0.2 ml of 0.05 M buffer (Tris–HCl pH 8.6) and 0.1 ml of suitably diluted enzyme were added to a final volume of 2.0 ml and the reaction mixture was incubated for 10 min at 37 °C. After the incubation period the reaction was stopped by adding 0.5 ml of 1.5 M trichloroacetic acid (TCA). To 0.5 ml of the above reaction mixture 6.5 ml distilled water and 1.0 ml Nessler's reagent were added and incubated for 10 min. The OD was measured at 480 nm. The blank was run by adding enzyme preparation after the addition of TCA. One unit (U) of L-asparaginase is defined as the amount of enzyme which liberated 1 μmol of ammonia/min/ml under the above assay conditions. Specific activity was given as units per milligram protein.

Asparaginase assay was also performed for the formation of β-aspartyl hydroxamate. The reaction mixture in a total volume of 4 ml contained 10 mM L-asparagine, 100 mM hydroxylammonium sulphate (adjusted to pH 6.3 with 100 mM KOH), 15 mM sodium phosphate buffer (pH 6.3) and suitably diluted enzyme. This was incubated at 37 °C for 30 min. To the enzyme reaction product, β-aspartyl hydroxamate ferric chloride reagent (5%) was added and the brown colored complex with β-aspartyl hydroxamate was measured at 500 nm. Controls were run by adding the enzyme after the addition of ferric chloride reagent at the end of the incubation period. Authentic β-aspartyl hydroxamate (Sigma) was employed as standard. One unit of asparaginase is defined as the amount of enzyme that formed 1 μmol of β-aspartyl hydroxamate in 1 min. The specific activity is expressed as units of asparaginase per milligram protein.

2.8. Experimental design and optimization

A central composite rotatable design (CCRD) with 5 variables was followed to examine the response pattern and to determine